Separation and Properties of Potato Invertase and Invertase Inhibitor

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A procedure involving vigorous blending of protein preparations has been developed for the separation of an endogenous macromolecular inhibitor from potato invertase. The resultant foaming completely destroys the inhibitor but does not inactivate the enzyme. Application of this technique revealed that invertase in potato tubers is always detectable but is present in highest levels in tubers stored at low temperatures. A marked decrease in invertase and a concomitant increase in inhibitor occur when tubers are transferred from cold to warm storage. Both the enzyme and its inhibitor have been partially purified. Optimal invertase activity was shown to occur at pH 4.7. Addition of purified inhibitor to invertase produces noncompetitive inhibition. The inhibitor is most effective at the pH optimum for invertase and its presence results in double pH optima.

Studies on invertase in potato tubers have indicated low and sporadic levels of activity (1–3). Among the factors which have been reported to influence invertase activity in tubers are the occurrence of sprouting (4), elemental nutrition of the plant (5), and the temperature during storage (6). Recently, Schwimmer *et al.* (1, 6) have obtained evidence that potato tubers contain a nondialyzable inhibitor which accompanies the enzyme in the preparation of crude extracts. The unusually low and variable invertase activity can be attributed, at least in part, to the presence of this endogenous inhibitor. The probable presence of variable levels of inhibitor in enzyme preparations described in the literature accounts for the contradictions concerning the properties of potato tuber invertase (2).

The present paper describes a procedure for separation of the inhibitor from invertase. This method was applied to study the variation of invertase and inhibitor in potato tubers. Purification of the two components and their properties, with emphasis on the effects of inhibitor on invertase, are reported.

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MATERIALS AND METHODS

Chemicals. All chemicals were reagent grade. The Sephadex G-100, calcium phosphate, and alumina C_γ gels were purchased from the Sigma Chemical Co., St. Louis, Missouri.[2]

Potato tubers. We used two varieties of potatoes, Kennebec and Pontiac, grown in 1964 on the Research Farm of the Red River Valley Potato Growers' Association. The mature tubers were stored continuously at 4°C since harvest and were transferred to 18°C as required. The storage rooms were dark and constant temperature was maintained with a good circulation of air.

Invertase assay. The assay procedure for invertase involved incubation of sucrose with the enzyme preparation in the presence of acetate buffer. The glucose and fructose formed were measured quantitatively by the Nelson method (7). At the enzyme concentrations employed in

[2] Reference to certain products or companies does not imply an endorsement by the Department over others mentioned.

the routine assay, the reaction was linear with time throughout the incubation period.

The incubation mixture contained 400 μmoles of sodium acetate buffer, pH 4.7, 730 μmoles sucrose, and a suitable aliquot of invertase preparation in a total volume of 5 ml. The samples, including a heated enzyme control, were incubated at 37°C for 1 hour. The reactions were terminated by addition of 5 ml of 0.5 *M* dibasic sodium phosphate and heating in a boiling water bath for 2 minutes. One ml of the solution was then analyzed for reducing sugars by heating with 1 ml of copper reagent in a boiling water bath for 20 minutes. The solution was cooled and 1 ml of arsenomolybdate reagent was added, the sample was diluted, and the optical density was measured at 520 mμ. A unit of invertase is defined is that amount of enzyme which catalyzes the liberation of 1 μmole of reducing hexose (glucose + fructose) per hour under the conditions of the assay.

Invertase inhibitor assay. Invertase inhibitor was assayed by measuring the reduction in rate of reaction on addition of inhibitor to 1 unit of purified invertase. At least three different amounts of inhibitor preparation were added to a constant amount of invertase and the rate of reaction was determined. A plot of $1/v$ against inhibitor concentration (8) yielded a straight line from which the amount of inhibitor necessary to decrease the rate of reaction by 50% was estimated. A unit of inhibitor is defined as that amount which inhibits one unit of invertase 50% at pH 4.7. Specific activity is defined as inhibitor units per milligram protein.

Protein determination. Protein was determined by the biuret method (9) in relatively crude extracts, and by absorbance readings at 280 mμ (10) in more purified and dilute extracts; crystalline bovine serum albumin was used as a standard.

Preparation of crude extracts. The tubers were peeled, cut into thin slices, and homogenized by dropping against the blades of a Waring Blendor running at low speed. The slurry was squeezed through several layers of cheesecloth and the juice obtained was clarified by centrifugation at 10,000*g* for 20 minutes. Sodium sulfite was added to a concentration of 0.01 *M* to prevent darkening of the supernatant solution. The solution was then dialyzed against 20 volumes of 0.1 *M* NaCl at 4°C with several changes in dialyzing solution. A small amount of precipitate which formed during dialysis was removed by centrifugation.

Purification of invertase. An extract was prepared from 500 gm of Kennebec tubers stored at 4°C for 24 weeks. The dialyzed solution was blended at high speed for a total of 30 minutes. The blending operation was interrupted at 5-minute intervals to cool the solution to 25°C by immersing the blender jar in ice water. During each 5-minute blending period the temperature increased to about 40°C. The blended solution was centrifuged and acidified to pH 4.5 by addition of dilute acetic acid. The precipitate that formed on warming the solution to 37°C was removed and discarded. Subsequent steps were carried out at 4°C.

The supernatant solution was then chromatographed in 50-ml portions on a 4 × 45 cm column of Sephadex G-100 washed with 0.1 *M* sodium acetate buffer, pH 4.5, containing 0.1 *M* NaCl and 10^{-3} *M* mercaptoethanol. Invertase was eluted by this solution as a sharp peak distinctly ahead of the major protein peak.

Further purification was achieved by adsorption on alumina C_γ gel. The eluates containing invertase from the Sephadex column were combined and dialyzed against 0.1 *M* NaCl. The pH was adjusted to 6.0 by addition of dilute NaOH and alumina C_γ gel was added until all the invertase was adsorbed. The gel was then washed three times with 0.1 *M* sodium phosphate, pH 6.0, and the enzyme was eluted with 0.2 *M* phosphate buffer, pH 6.0, containing 0.2 *M* NaCl. The eluate was dialyzed against 0.1 *M* NaCl containing 10^{-3} *M* mercaptoethanol, acidified to pH 4.5, and stored at 3°C. A summary of the purification is presented in Table I.

Purification of invertase inhibitor. All operations were carried out at 4°C. A kg of Pontiac tubers that had been stored at 4°C for 18 weeks

TABLE I

PURIFICATION OF INVERTASE FROM POTATO TUBERS

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
1. Crude extract	210	1890	567	0.3	—
2. After blending for 30 minutes	190	1650	8500	5.2	100
3. pH 4.5 step	195	1420	8240	5.8	97
4. Sephadex G-100 eluate	200	138	5680	41.2	67
5. Alumina C_γ gel eluate	50	63	4430	70.4	52

TABLE II

PURIFICATION OF INVERTASE INHIBITOR FROM POTATO TUBERS

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
1. Crude extract	310	2360	9670	4.1	100
2. pH 4.5 step	320	2120	9550	4.5	99
3. Alumina C_γ gel eluate	50	330	6770	20.5	70
4. Calcium phosphate gel eluate	50	125	6100	48.7	63

and subsequently at 18°C for 6 weeks was used. During the extraction procedure, special precautions were taken to avoid excessive foaming. The dialyzed solution was acified to pH 4.5 and the precipitate was removed and discarded. The pH of the supernatant solution was adjusted to 5.5 by careful addition of dilute NaOH. Alumina C_γ gel was then added at the rate of 0.1 gm solids per 100 ml solution. This level of alumina C_γ gel adsorbed most of the inhibitor and only about 20% of the protein. The alumina C_γ gel was collected by slow centrifugation and washed three times with 0.05 *M* phosphate buffer, pH 5.5. Elution of the inhibitor was accomplished with 0.2 *M* phosphate buffer, pH 5.5, containing 0.5 *M* NaCl. The eluates were combined and dialyzed against 0.1 *M* NaCl.

The solution was adjusted to pH 6.0 by addition of dilute NaOH and 0.5 gm solids of calcium phosphate gel was added. About half the protein and all of the inhibitor were adsorbed on the gel. After washing the calcium phosphate three times with 0.05 *M* phosphate buffer, pH 6.0, the inhibitor was eluted with 0.2 *M* phosphate buffer, pH 6.0, containing 0.2 *M* NaCl. The solution was dialyzed against 0.1 *M* NaCl and acidified to pH 4.5. Purification of the inhibitor is summarized in Table II. Invertase inhibitor prepared by this procedure was free of invertase. Tests of homogeneity for the partially purified inhibitor and invertase were not performed, but it is likely that the preparations were still contaminated by other proteins.

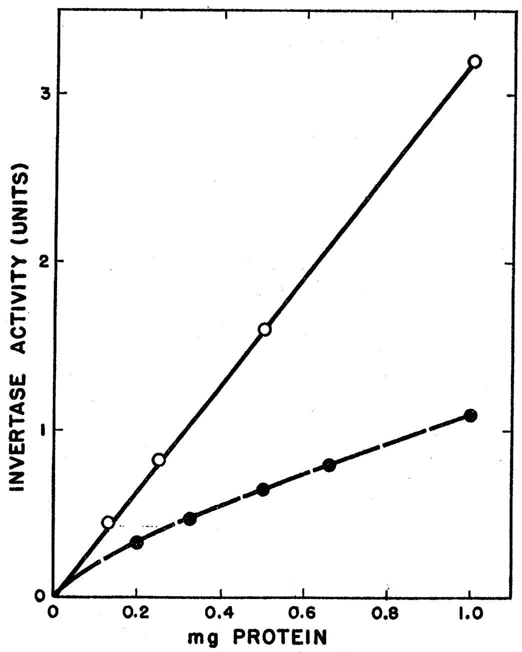

FIG. 1. Effect of foaming on the relationship between enzyme concentration and rate of reaction for potato invertase. Activity is expressed as μmoles of reducing sugars formed per hour. The assay conditions are described in the text. ●—● Crude extract of potato tubers before blending; ○—○ the same extract after blending for 30 minutes.

RESULTS

Effect of Foaming on Invertase Activity in Crude Extracts

Preliminary examination of invertase in potato tubers revealed that the activity is influenced by the mode of preparation of enzyme extracts. Very low invertase activities were obtained when the extracts were prepared by procedures involving low temperature and minimum foaming. Moreover, the relationship between protein concentration and rate of enzyme action for these invertase preparations deviated from linearity (Fig. 1). Highest activities were obtained when extracts were prepared by prolonged high speed blending at room temperature. When sufficient blending was employed, the invertase prepared in this manner exhibited linearity between protein concentration and rate of reaction (Fig. 1). These observations led to the discovery that the invertase inhibitor in potato tubers

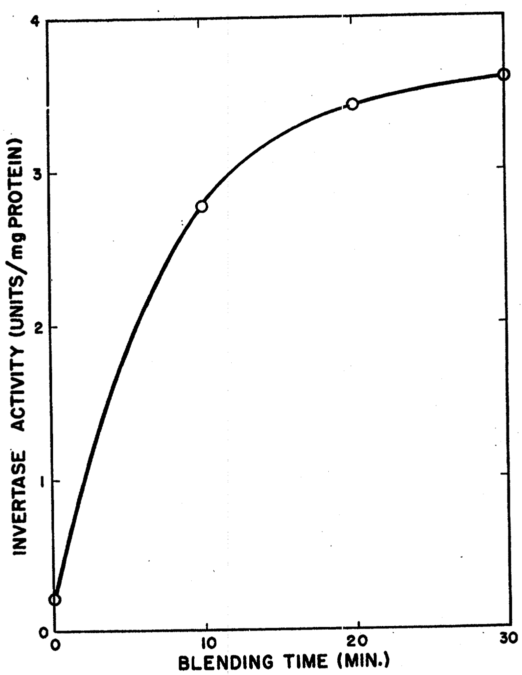

FIG. 2. Effect of foaming time on invertase activity in an extract of potato tubers. The sample was blended for 5-minute intervals and invertase was determined as described in the text.

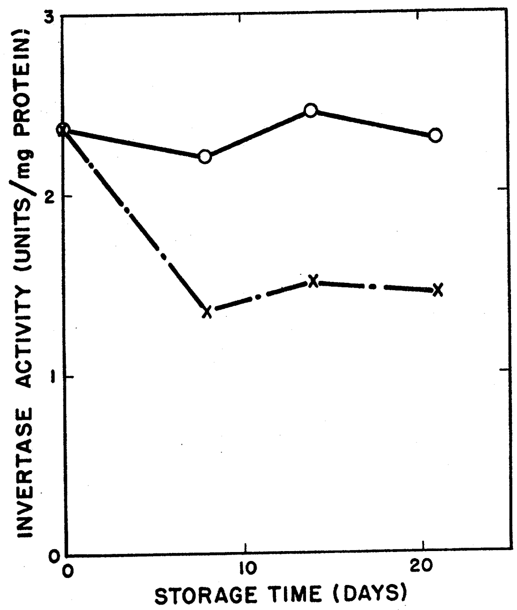

FIG. 3. Effect of storage temperature on invertase in potato tubers. Invertase activity was measured after the destruction of the invertase inhibitor by the routine assay. O—O Extracts prepared from tubers stored at 4°C; X—X extracts prepared from tubers stored at 18°C.

can be surface denatured without inactivation of invertase.

The effect of foaming as achieved by vigorous blending on invertase activity in an extract is shown in Fig. 2. This extract was prepared by blending the potato slices very briefly at 3°C to minimize foaming effects during preparation. A 200-ml portion of the dialyzed extract was then blended for 5 minutes and cooled to 25°C. This procedure was repeated for a total blending time of 30 minutes. Aliquots of the solution were removed after various blending times and assayed for invertase. For this particular tuber sample, the invertase activity in the unblended extract was only 0.22 unit per milligram protein. After blending the solution for 30 minutes, the activity increased 16 times. The invertase activity in every extract studied was increased by foaming.

The 30-minute blending time was sufficient to destroy the inhibitor in most extracts. Blending for longer periods did not result in reduced invertase activity, indicating that the enzyme is stable to this treatment. The blending procedure was a convenient method for the destruction of the inhibitor. However, foaming of extracts by other methods, including passage of nitrogen through the solutions, were equally effective.

Variation of Invertase and Invertase Inhibitor

Invertase activity in unblended extracts of potato tubers was generally low and often undetectable. The actual activity in extracts of tubers stored at 4°C, as determined after destruction of the inhibitor, ranged from 2 to 7 units per milligram protein. The highest activities observed are comparable to the invertase in washed slices of red beet and sugar beet roots and Jerusalem artichoke tubers, but considerably lower than the activity which develops in slices of chicory roots (11, 12). In contrast to the insolubility of the invertase in these storage tissues, the invertase in potato tubers is readily soluble.

Storage temperature has a great influence on invertase and inhibitor levels in tubers. Figure 3 shows the results of a study on the effect of storage temperature on invertase in

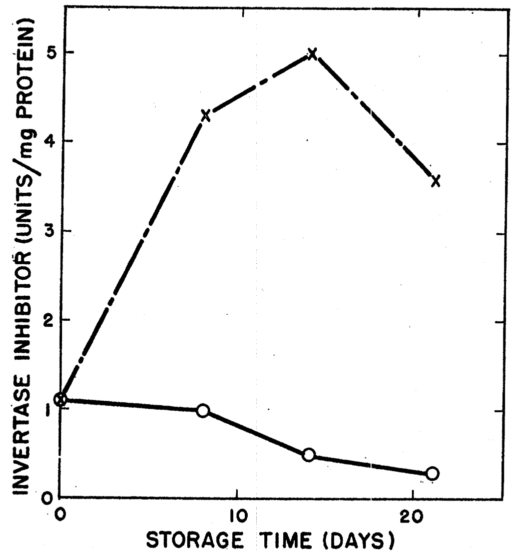

FIG. 4. Effect of storage temperature on invertase inhibitor in potato tubers. O—O Extracts prepared from the tubers stored at 4°C; X—X extracts prepared from tubers stored at 18°C.

Pontiac tubers. One-half of the sample which had been stored at 4°C for 31 weeks was left at 4°C and the other half was transferred to storage at 18°C. Invertase was measured in blended extracts at regular intervals. The activity remained essentially constant in the tubers at 4°C during this period. In contrast to the small changes at 4°C invertase decreased by one-third in the tubers at 18°C during the first week and then remained constant.

The unblended extracts of both series of samples were assayed for excess invertase inhibitor (Fig. 4). The level of inhibitor decreased slightly in the tubers at 4°C but increased 4 times durng the first week in the tubers at 18°C.

The marked and rapid changes in invertase and excess inhibitor were always observed when tubers were transferred from cold to warm storage. Accompanying these changes was a large reduction in reducing sugars which were determined by the Nelson method (7) in alcoholic extracts of the tubers. Determination of total inhibitor in extracts was not possible because invertase could not be separated without considerable loss of inhibitor. Therefore, it was not established if total inhibitor varied during storage. The observed changes in excess inhibitor could be attributed to variation in invertase rather than changes in the level of total inhibitor.

Sprouting of tubers occurred after several weeks of storage at 18°C. Analysis of a few sprout samples indicated an invertase level in the range of 7–10 units per milligram protein. It was found that the invertase was completely free of inhibitor. Thus, crude extracts exhibited linearity between enzyme concentration and rate of reaction and the activity was not affected by foaming. However, invertase preparations from sprouts were inhibited by tuber inhibitor, although about 2 units of inhibitor were required to reduce the activity of one unit invertase to 50%.

Properties of Invertase Inhibitor

The inhibitor possesses properties characteristic of proteins. The ratio of carbohydrate, as measured by the Anthrone method (13), to protein was only 1:450 for the partially purified inhibitor. The inhibitor was nondialyzable and was insoluble in 35% ammonium sulfate. It was adsorbed readily on both calcium phosphate and alumina Cγ gels. The inhibitor was eluted off a column of Sephadex G-100 only slightly later than the invertase peak.

In addition to inactivation by foaming, the inhibitor was thermolabile with denaturation above 50°C. The inhibitor was sensitive to alkaline pH with 50% loss of activity at pH 7.0 and 3°C overnight. No loss of inhibitor occurred at pH 4.5 after one month at 3°C.

Effect of Invertase Inhibitor on the Properties of Invertase

pH optimum. The reaction for invertase in the absence of its inhibitor proceeded at maximum velocity in the pH range of 4.5–5.0 (Fig. 5). The actual optimum therefore is at a pH somewhat higher than that predicted from studies on invertase preparations containing the inhibitor (6) and is slightly lower than that reported recently (2). The reaction was not influenced by the nature of the buffer. Activation by calcium (2) was not confirmed.

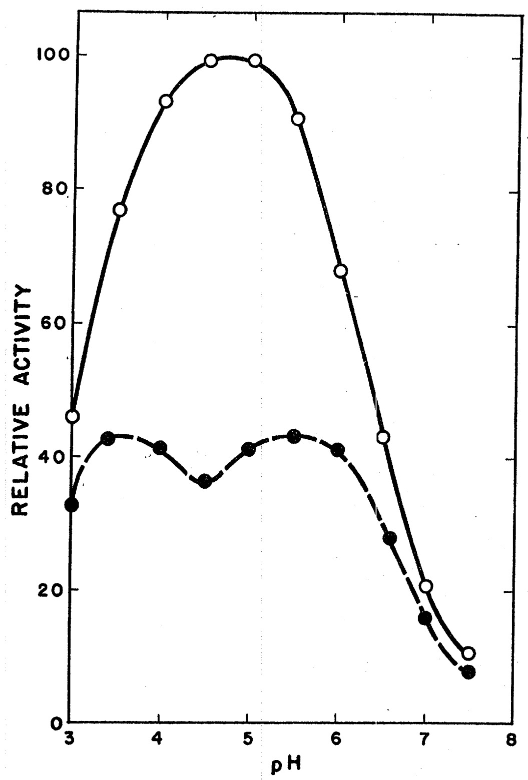

FIG. 5. Effect of pH on invertase activity. The buffers used were 0.04 *M* citrate in the pH 3.0–5.5 range and 0.04 *M* phosphate in the pH 6.0–7.5 range. Activity was determined by the routine assay with sucrose as substrate. ○—○, 15 units of purified invertase in the absence of inhibitor; ●—● 15 units of invertase in the presence of 20 units of inhibitor.

Addition of invertase inhibitor to the enzyme had a great effect on the pH dependence curve (Fig. 5). The inhibitor reduced reaction velocity most effectively near the pH optimum. The inhibition decreased as the pH was varied in either direction from the optimum. Double pH optima with a minimum at about pH 4.5 were always observed for invertase solutions containing the inhibitor. This minimum in the curve was especially pronounced at high relative concentrations of inhibitor.

Stability. Partially purified invertase at pH 4.5 was completely inactivated by heating at 65°C for one minute. However, the stability of invertase was greatly enhanced in the presence of the inhibitor. For example, 50% of the enzyme was recovered after a solution of invertase at pH 4.5 containing a sufficient amount of inhibitor to completely inhibit its action was heated to 70°C for 5 minutes. This is in accord with observations that the invertase in crude extracts prior to destruction of the inhibitor was unusually stable to heat.

Effect of substrate concentration. Figure 6 shows the effect of sucrose concentration on the rate of reaction for inhibitor-free invertase and invertase in the presence of two levels of inhibitor. These data are replotted according to the method of Lineweaver and Burk (14) in Fig. 7. The Michaelis constant for potato invertase was calculated to be 6.1×10^{-3} *M*. It is clear that inactivation of invertase by the inhibitor depends solely on the concentration of the inhibitor and the maximal reaction velocity attained is less than that found in the absence of inhibitor at all substrate concentrations. The invertase inhibitor therefore functions as a pure noncompetitive enzyme inhibitor.

DISCUSSION

The existence of an invertase system in potato tubers consisting of the enzyme and an inhibitor (1, 6) was confirmed. A marked increase in invertase activity and the change to a linear relationship between protein concentration and rate of reaction after foaming of extracts are evidence that the inhibitor is surface denatured. This technique, therefore, is a simple method for the preparation of inhibitor-free invertase and provides an assay for total invertase in potato tubers.

The physiological significance of not only the invertase but also its inhibitor is a subject for speculation. Opinions concerning the biological role of invertase vary considerably. Straus (15) observed that there is no quantitative correlation between invertase activity and the rate of growth of tissue cultures. However, invertase activity is usually associated with rapid growth (16, 17) although more efficient pathways exist for utilization of translocated sucrose.

The role of invertase in mature storage tissue is not related to growth as increase in the number and size of cells does not occur. Moreover, during sprouting of potato tubers

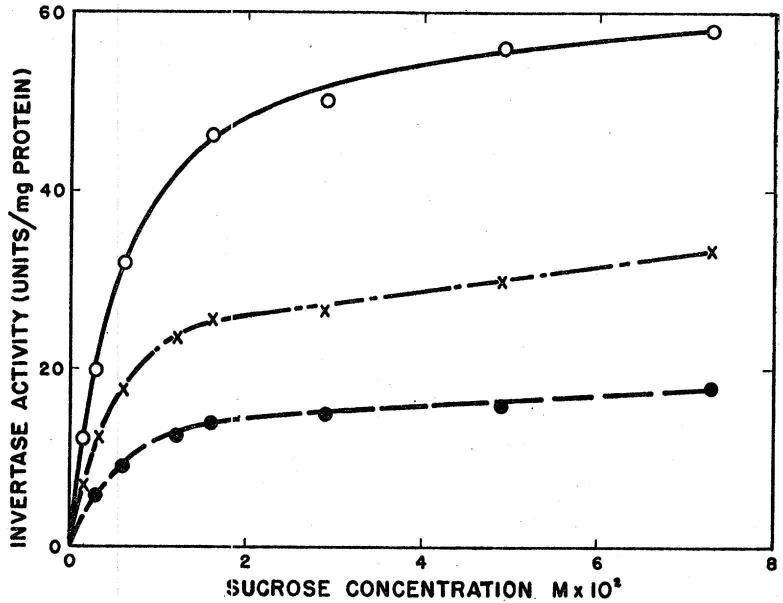

FIG. 6. Effect of sucrose concentration on the rate of invertase action. The level of purified invertase was 15 units for all three curves. ○—○ Invertase; ×—× invertase in the presence of 15 units of invertase inhibitor; ●—● invertase in the presence of 30 units of inhibitor. The samples were made up to a total volume of 5 ml, and standard incubation conditions were used.

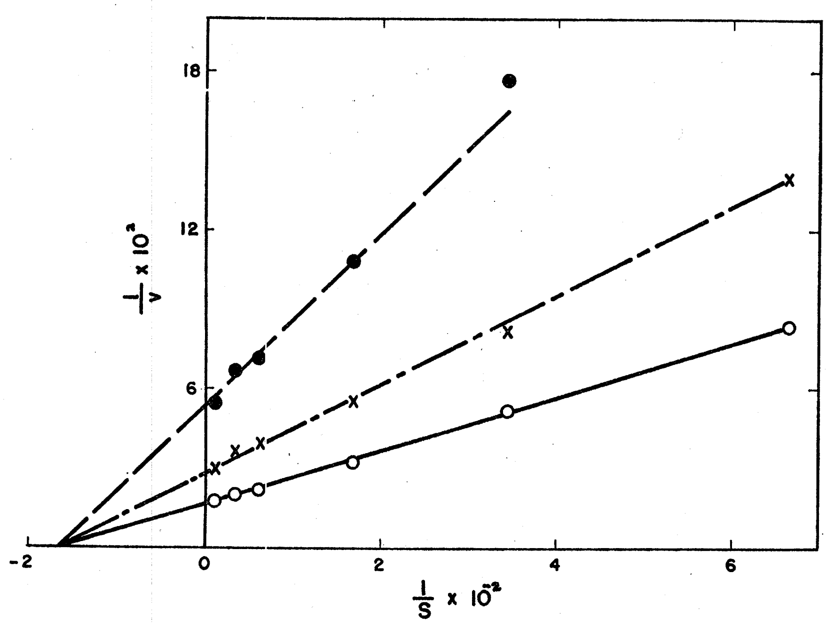

FIG. 7. Lineweaver-Burk plots for invertase showing noncompetitive inhibition by invertase inhibitor. ○—○ 15 units of invertase; ×—× 15 units of invertase in the presence of 15 units of inhibitor; ●—● 15 units of invertase in the presence of 30 units of inhibitor.

when conversion of storage carbohydrate proceeds most rapidly invertase activity is very low. Highest activity is observed in tubers at low temperature which also leads to accumulation of reducing sugars (18). Cold hardiness of plants has been related to the sugar content (19) among other factors. If a high soluble sugar content does protect plant tissue from frost damage, a means for regulating the sugar level must exist. The function of invertase in such a mechanism would be to hydrolyze sucrose to reducing sugars to provide a high concentration of soluble sugars in response to low temperature.

Invertase activity in potato tubers appears to be regulated in two ways. The enzyme itself fluctuates considerably in response to storage conditions. In addition, the enzyme is under the influence of an inhibitor which may also vary according to conditions. Usually a high level of invertase is present in the tubers, but its activity is reduced to almost zero by the inhibitor. When the temperature is lowered the basal invertase activity is increased by synthesis of new enzyme and by reduction in the level of inhibitor.

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